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Chick Pineal Melatonin Synthesis: Light and Cyclic AMP Control Abundance of Serotonin *N*-Acetyltransferase Protein

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Abstract: Melatonin production in the pineal gland is high at night and low during the day. This rhythm reflects circadian changes in the activity of serotonin *N*-acetyltransferase [arylalkylamine *N*-acetyltransferase (AA-NAT); EC 2.3.1.87], the penultimate enzyme in melatonin synthesis. The rhythm is generated by an endogenous circadian clock. In the chick, a clock is located in the pinealocyte, which also contains two phototransduction systems. One controls melatonin production by adjusting the clock and the other acts distal to the clock, via cyclic AMP mechanisms, to switch melatonin synthesis on and off. Unlike the clock in these cells, cyclic AMP does not appear to regulate activity by altering AA-NAT mRNA levels. The major changes in AA-NAT mRNA levels induced by the clock seemed likely (but not certain) to generate comparable changes in AA-NAT protein levels and AA-NAT activity. Cyclic AMP might also regulate AA-NAT activity via changes in protein levels, or it might act via other mechanisms, including posttranslational changes affecting activity. We measured AA-NAT protein levels and enzyme activity in cultured chick pineal cells and found that they correlated well under all conditions. They rose and fell spontaneously with a circadian rhythm. They also rose in response to agents that increase cyclic AMP. They were raised by agents that increase cyclic AMP, such as forskolin, and lowered by agents that decrease cyclic AMP, such as light and norepinephrine. Thus, both the clock and cyclic AMP can control AA-NAT activity by altering the total amount of AA-NAT protein. Effects of proteosomal proteolysis inhibitors suggest that changes in AA-NAT protein levels, in turn, reflect changes in the rate at which the protein is destroyed by proteosomal proteolysis. It is likely that cyclic AMP-induced changes in AA-NAT protein levels mediate rapid changes in chick pineal AA-NAT activity. Our results indicate that light can rapidly regulate the abundance of a specific protein (AA-NAT) within a photoreceptive cell. **Key Words:** Pineal—Circadian rhythms—Proteosomal proteolysis—Cyclic AMP—Melatonin—Photoreceptors—Serotonin *N*-acetyltransferase.
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In all vertebrate classes, synthesis and release of the hormone melatonin are high at night and low in the daytime (Klein et al., 1997). Melatonin is the hormone of the pineal gland (Arendt, 1995), although it is also synthesized in retina (Cahill and Besharse, 1995; Iuvone et al., 1997). The best established function of melatonin in mammals is to mediate the major seasonal changes in physiology and behavior shown by photoperiodic species (Goldman and Nelson, 1993). In addition to its role in photoperiodism, melatonin has been shown capable of acting as a feedback regulator of the circadian pacemaker in the mammalian brain (Cassone, 1990).

The rhythmic pattern of melatonin synthesis reflects the activity of serotonin *N*-acetyltransferase [arylalkylamine *N*-acetyltransferase (AA-NAT); EC 2.3.1.87]. Mammalian AA-NAT activity increases at night by as much as 100-fold, depending on the species. This represents one of the most dramatic and highly conserved hormonal and biochemical rhythms in vertebrates. AA-NAT activity is regulated by multiple mechanisms. These differ among vertebrates, but certain features are highly conserved. One is that an endogenous circadian clock drives the rhythms of AA-NAT activity and melatonin synthesis. A second is that light typically acts on the system in two ways: the first is to reset (entrain) the clock, and the second is to act more directly on AA-NAT to rapidly switch off its activity. The rapid effects of light appear to involve cyclic AMP (cAMP) (Klein et al., 1998).

There are important differences in the organization of photic and circadian regulatory mechanisms between mammals and birds. In mammals, the pineal gland does not itself contain a circadian clock, nor is it directly photosensitive. The circadian rhythm in pineal AA-NAT activity in mammals is driven by a circadian clock located in the brain, specifically, in the suprachiasmatic nucleus (SCN) of the hypothalamus (Klein et al., 1990).

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Abbreviations used: AA-NAT, arylalkylamine *N*-acetyltransferase, serotonin *N*-acetyltransferase; cAMP, cyclic AMP; L, white light; LR 12:12, 12-h white light/12-h red light; R, red light; RR, constant red light; SCN, suprachiasmatic nucleus; ZT, zeitgeber time.

Information from the SCN is transmitted to the rat pineal gland by a multisynaptic neural pathway terminating in adrenergic fibers that release norepinephrine at night, stimulating melatonin synthesis. Light acts on the SCN via retinal photoreceptors and a retinohypothalamic projection. Exposure to light at night in vivo rapidly blocks adrenergic stimulation of the gland, which rapidly decreases cAMP and melatonin synthesis.

In contrast, the chick pineal contains within itself a complete and independent circadian system: photoreceptors, a circadian pacemaker, and the apparatus for melatonin synthesis (Zatz, 1996). In vivo the chicken pineal rhythm is regulated by two oscillators, one in the pineal gland itself (Deguchi, 1979*a*; Kasal et al., 1979; Binkley, 1988) and another in the avian homologue of the SCN (Cassone, 1990). However, the clock in the pineal is sufficient to generate a rhythm in AA-NAT activity even in dispersed cell culture (Deguchi, 1979*a*; Kasal et al., 1979; Zatz et al., 1988; Takahashi et al., 1990). Consequently, melatonin output in vitro is several times higher at night than in the day, and persists (although it damps) in constant darkness or constant red light. Furthermore, chick pineal cells are directly photosensitive, which makes it possible for light to act on these cells in vitro to acutely suppress nocturnal melatonin synthesis and also to entrain the pineal circadian clock. The acute inhibitory effect of light appears to be mediated by cAMP, whereas the entraining effect of light is not (Zatz and Mullen, 1988*a*,*b*). A third difference is that, in the chick, norepinephrine is released during the day and inhibits (rather than stimulates, as in mammals) the synthesis of melatonin (Deguchi, 1979*b*; Cassone and Menaker, 1983).

Analysis of the regulation of avian AA-NAT mRNA levels and enzyme activity has revealed that cAMP has relatively small effects on AA-NAT mRNA, which is controlled primarily by the pineal clock. In contrast, cAMP has profound effects on AA-NAT enzyme activity (Bernard et al., 1997*b*) and appears to act downstream from the rhythmic changes in AA-NAT mRNA. Likewise, it appears that the main factor mediating the light-induced decrease in AA-NAT activity is a decrease in cAMP, because light has little rapid influence on AA-NAT mRNA.

In the present investigation, we examined the relationship between changes in AA-NAT enzyme activity and changes in AA-NAT protein levels in chick pineal cells. Results indicate a strong relationship, as has been seen in mammals (Klein et al., 1997; Gastel et al., 1998). We determined whether the changes in mRNA levels induced by the clock are indeed reflected in changes in AA-NAT protein levels, and whether, despite the small effect of changes in cAMP on mRNA levels, its effects on enzyme activity are still caused by changes in AA-NAT protein levels, rather than by some other mechanism such as posttranslational modifications or interactions with other proteins. Results indicate that both the clock and cAMP affect enzyme activity by changing AA-NAT protein levels. This effect of cAMP is similar to that seen in mammals (Klein et al., 1997; Gastel et al., 1998). Furthermore, cAMP appears to act by controlling the rate at which AA-NAT protein is destroyed by proteosomal degradation. We suspect that this mechanism is primarily responsible for the rapid effects of light on chick pineal AA-NAT activity, as seems also to be the case in mammals in vivo (Gastel et al., 1998). These results provide a clear example of how light can selectively control the abundance of a specific protein.

MATERIALS AND METHODS

Pineal cell culture

Cell preparation and culture. White leghorn chicks were received 0–2 days after hatch from Truslow Farms (Chestertown, MD, U.S.A.). Pineal cells were dispersed in trypsin and plated in modified McCoy's 5A medium (GIBCO 12330-023) containing 25 m*M* HEPES buffer, L-glutamine, penicillin, streptomycin, 10% heat-inactivated fetal bovine serum, and 10% chicken serum as described previously (Zatz et al., 1988). Experiments used cells from up to 150 glands plated with $\sim 3 \times 10^6$ cells/well into six-well plates. Plates contained one to three groups of two wells each. Days in culture are numbered successively from the day of plating (day 1). Cultures were fed by exchange of medium at least daily. Serum-containing media were used through day 3, and serum-free media with 10 m*M* KCl added were used from day 4 onward. The effects of feeding schedule, media, sera, and potassium have been described (Zatz et al., 1988).

Light cycles, drugs, and harvests. Cells were maintained at 37°C under 5% CO_2 in air in tissue culture incubators containing red lights, white lights, and timers as described previously (Zatz et al., 1988). They were all exposed to a cycle of 12-h white light (L) and 12-h red light (R) (LR 12:12) through day 5. In this schedule, L acts as "day" and, by convention, starts at zeitgeber time (ZT) 0; R acts as "night" and starts at ZT 12. Entrainment schedules varied in their relationship to "solar" time; groups of cells within each experiment were often entrained to opposite schedules to permit "midday" and "midnight" harvests to be performed at essentially the same time. In some experiments, groups of cells were switched from LR 12:12 to constant red light (RR) before expected onset of L at the start of day 6. Media were exchanged in L or R, as appropriate, so as not to disturb the lighting regimen.

Forskolin (Sigma, St. Louis, MO, U.S.A.), nitrendipine (RBI, Natick, MA, U.S.A.), and Bay K 8644 (RBI) were dissolved in alcohol and diluted 1:1,000 into medium. MG 132 (Calbiochem, La Jolla, CA, U.S.A.) was dissolved in dimethyl sulfoxide and diluted 1:1,000 into medium. Cycloheximide (Sigma), lactacystin (Calbiochem), and calpain inhibitor I (Calbiochem) were dissolved in water and diluted 1:100 or 1:1,000 into medium. Norepinephrine (Sigma) was dissolved in 10 mg/ml ascorbate and diluted 1:100 into medium. 8-Bromo-cAMP (RBI) was dissolved directly into medium.

Cells were harvested by scraping in phosphate-buffered saline and then centrifuged. Cell pellets were stored at −70°C. The cell pellets were resuspended, disrupted by sonication, and centrifuged to separate supernatant from membranes. Samples of supernatant were taken for immunoprecipitation, assay of AA-NAT enzyme activity, and determination of total protein content. Generally, material from one well of a six-well plate ultimately provided the sample for one gel lane. In other experiments, initial cell suspensions were plated into six-well plates for determination of AA-NAT protein and into 12-well plates for subsequent determination of AA-NAT enzyme ac-

tivity. Generally, material from one well of a 12-well plate provided the sample for one AA-NAT activity assay.

AA-NAT enzyme activity assay

Duplicate aliquots of supernatant were assayed for AA-NAT enzyme activity using [^{3}H]acetyl-CoA and tryptamine substrates as described previously (Bernard et al., 1997*b*). Reactions were stopped with HCl, and acetylated product was extracted into chloroform. The extract was washed with NaOH solution to reduce blank, and an aliquot was dried and counted.

AA-NAT immunoprecipitation, phosphorylation, and assay

AA-NAT protein was assayed, following immunoprecipitation, by phosphorylation and subsequent polyacrylamide gel electrophoresis to resolve the relevant bands for quantification (Gastel et al., 1998). Samples were immunoprecipitated at 4°C (rotating) for 2 h in 200 μl of immunoprecipitation buffer containing 500 m*M* NaCl, 50 m*M* Tris, pH 8.0, 1 m*M* fresh dithiothreitol, 100 μ*M* fresh phenylmethylsulfonyl fluoride, and 2 μl of undiluted Ab 2822. Ab 2822 is a polyclonal antiserum raised against purified bacterially expressed rat AA-NAT 50–150 fused to measles fusion protein 288–302 as described (Gastel et al., 1998), which immunoprecipitates chicken AA-NAT. Antibody-bound AA-NAT was captured using 25 μl of washed protein A-Sepharose CL-4B beads (Pharmacia, Piscataway, NJ, U.S.A.) at 4°C (rotating) for 1 h. Beads were separated by centrifugation and washed five times with immunoprecipitation buffer and once with protein kinase A buffer.

Immunoprecipitated enzyme samples were phosphorylated (1 h, 30°C) while still attached to the beads using excess protein kinase A catalytic subunit (Promega, Madison, WI, U.S.A.), ATP (Sigma), and [^{32}P]ATP (Amersham, Piscataway, NJ, U.S.A.). Reactions were stopped with gel loading buffer (Novex, San Diego, CA, U.S.A.). Samples were heated at 95°C for 10 min and then centrifuged to remove the beads, and supernatant samples were subjected to standard polyacrylamide gel electrophoresis on 15 cm × 0.75 mm 15% Duracryl gels (with stacking gel). Proteins were transferred from the gel onto polyvinylidene difluoride membrane (0.45 μm; Millipore Immobilon-P) in CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] buffer containing 20% methanol at 30 V overnight. Dried membranes were exposed to x-ray film, and radioactivity in the relevant bands was quantified by densitometry. "Volume" of appropriate bands, after subtracting blank, was computed using ImageQuant and a Molecular Dynamics 300A densitometer, and each measurement was normalized to the appropriate control group in the same experiment.

RESULTS

AA-NAT protein levels in cultured chick pineal cells were determined by a sensitive immunoprecipitation/radiolabeling method (Fig. 1). Two major bands were visible. They had apparent molecular masses of ~21.5 and 24.1 kDa, respectively. The expected molecular mass based on amino acid sequence is ~23 kDa. This is in contrast to results from rat pineal glands (Gastel et al., 1998), where a single band was detected. The relative abundance of these two bands did not vary significantly between experimental groups or in a distinct pattern between preparations; consequently, they were combined for purposes of quantification and comparisons of exper-

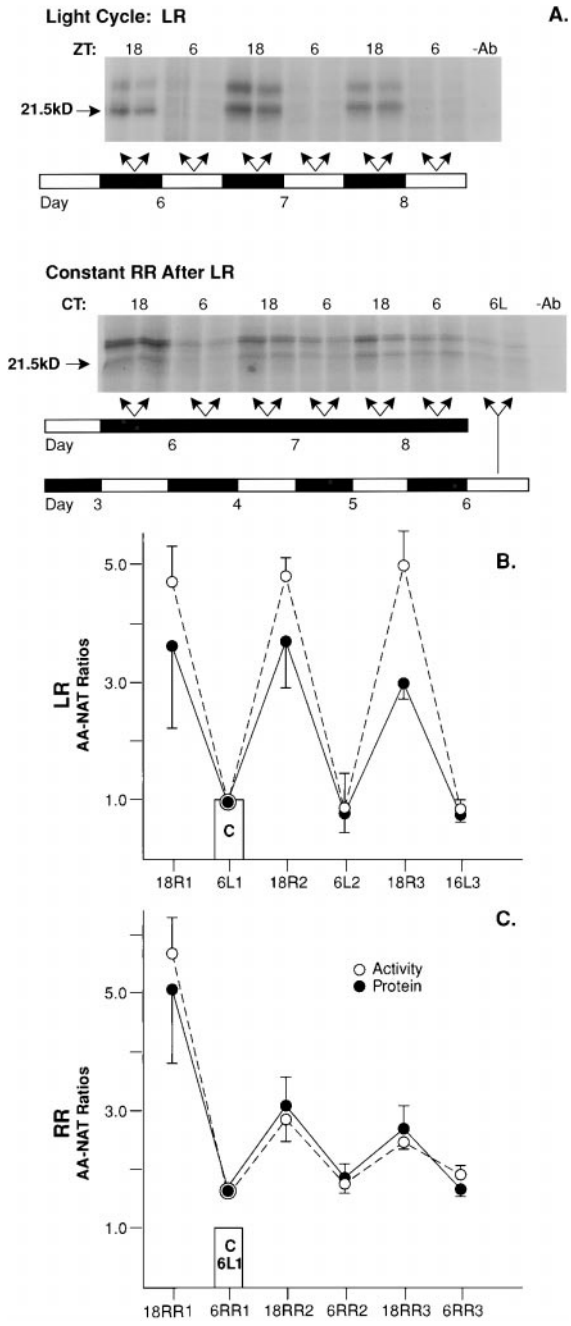

FIG. 1. Diurnal and circadian rhythms of AA-NAT protein levels and enzyme activity. **A:** Immunoprecipitates of duplicate samples were harvested at different times of day in an LR 12:12 cycle (**upper**) and in RR (**lower**). They were run on gels and visualized as described in Materials and Methods. Data from a typical experiment are shown. **B:** Protein levels and enzyme activity showed a similarly robust rhythm in LR. AA-NAT protein levels and enzyme activity were determined in cells harvested at different times of day in an LR 12:12 cycle. Results are means ± SE from four experiments, each in duplicate, normalized to the midday group (6L1, taken as 1.0). **C:** Protein levels and enzyme activity showed a similarly damped rhythm in RR. AA-NAT protein levels and enzyme activity were determined in cells harvested at different times of day in RR. Results are means ± SE from three experiments, each in duplicate, normalized to the midday group (6L1, not 6RR1).

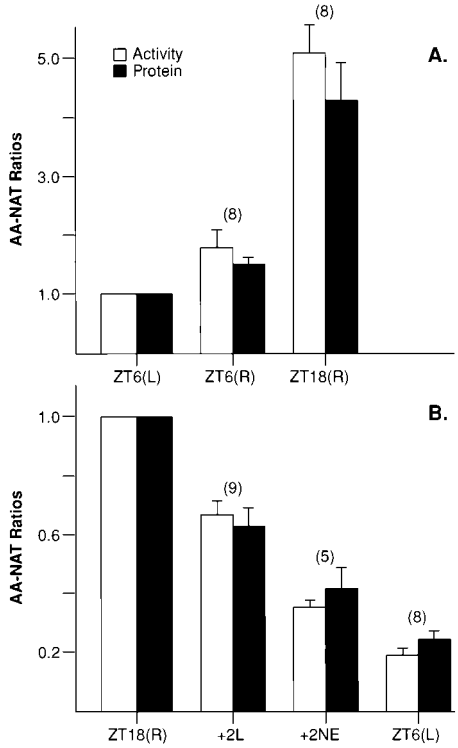

FIG. 2. Effects of changed lighting, and norepinephrine, on AA-NAT protein levels and enzyme activity. **A:** Protein levels and enzyme activity showed similar moderate increases in daytime R, relative to daytime L, and similar large increases at night. AA-NAT protein levels and enzyme activity were determined in cells harvested at midday (ZT 6) in light (L) and, after extension of red light (R) to midday in R, and at midnight (ZT 18) in R. Results are means ± SE from eight experiments, each in duplicate, normalized to the midday group [ZT6(L), taken as 1.0]. **B:** Nocturnal protein levels and enzyme activity showed similar moderate decreases in response to L at night, similar (greater) decreases to norepinephrine at night, and similar (large) decreases from night [ZT18(R)] to day [ZT6(L)]. AA-NAT protein levels and enzyme activity were determined in cells harvested at midnight (ZT 18) in R, or after 2 h of exposure to L (+2L) or to 10 μM norepinephrine (+2NE) from ZT 16 to 18. Results are means ± SE from the number of experiments shown in parentheses, each in duplicate, normalized to the midnight group [ZT18(R), taken as 1.0]. Also shown, for comparison, are the results from cells harvested at midday [ZT6(L)], taken as 1.0 in A.

imental groups. In the chick pineal cells, AA-NAT protein displayed a robust diurnal rhythm in an LR 12:12 cycle and a damped rhythm in RR (Fig. 1). These patterns are similar to those of melatonin production, AA-NAT mRNA levels, and AA-NAT enzyme activity seen in this system (Zatz et al., 1988; Bernard et al., 1997*a*,*b*).

Part of the damping in RR can be attributed to the loss of a "direct" suppressive effect of L. Daytime AA-NAT enzyme activity and protein levels are higher in R than in L (Fig. 2A, normalized to midday in L), as is known also to be the case for melatonin production (Zatz et al., 1988; Zatz, 1991). In nighttime R, when AA-NAT enzyme activity and protein levels are high, a 2-h pulse of L is sufficient to reduce nocturnal AA-NAT enzyme activity and protein levels considerably (Fig. 2B, normalized to midnight in R). L has a similar proportional effect at various day and night phases (data not shown). A stronger suppressive effect was seen with a 2-h pulse of 10 μM norepinephrine, which, as discussed in the introductory section, suppresses pineal melatonin synthesis in the chick (Deguchi, 1979*b*; Zatz and Mullen, 1988*b*) (Fig. 2B). A suppressive effect was also observed in response to 3 μM nitrendipine (Table 1), which does not alter AA-NAT mRNA in these cells (Bernard et al., 1997*b*), but does cause rapid reductions in melatonin production and AA-NAT activity (Zatz, 1992, 1996).

We tested the cAMP protagonist forskolin to determine if it could alter AA-NAT protein levels. Forskolin strongly increases melatonin production (Zatz and Mullen, 1988*b*) and AA-NAT enzyme activity, but does not alter AA-NAT mRNA levels much (Bernard et al., 1997*a*,*b*). We found that 2-h treatment of chick pineal cells with forskolin during the subjective night raised AA-NAT protein levels and enzyme activity in parallel (Fig. 3A). Similar (although proportionately larger) results were obtained with forskolin treatment during the day (Table 1). Nocturnal or daytime treatment with 8-bromo-cAMP or Bay K 8644 also increased AA-NAT protein levels and AA-NAT enzyme activity in parallel (Table 1). Treatment with forskolin also prevented or overcame the inhibitory effect of L at night (Fig. 3A). These results directly confirm the previous inference (Bernard et al., 1997*b*) that cAMP regulates acute changes in AA-NAT activity via posttranscriptional mechanisms (at translational or posttranslational levels). Furthermore, these mechanisms appear to be involved in translating increases in nocturnal AA-NAT mRNA into increases in AA-NAT enzyme activity.

TABLE 1. *Effects of drugs on AA-NAT protein levels and enzyme activity*

	ZT 6 (L)		ZT 18 (R)	
Group	Activity	Protein	Activity	Protein
ZT 6 (L) Control	1.0	1.0	—	—
ZT 18 (R) Control	—	—	1.0	1.0
FSK	4.0	3.0	2.4[a]	2.4[a]
8BA	4.0	3.7	2.6	2.2
MG	5.0	3.2	2.2[a]	2.8[a]
CPI	4.5	3.4	2.3	2.5
LTC	5.2	3.0	—	—
CHX	0.4	0.5	0.5	0.5
NTR	—	—	0.3	0.3
BK	1.9	1.8	1.4	1.6

AA-NAT protein levels and enzyme activity were determined in cells harvested at midday (ZT 6) in L or at midnight (ZT 18) in R. Results from experimental groups were normalized to these groups, taken as 1.0, respectively. Cells were exposed to drugs for 2 h before harvest. Drugs used were as follows: 1 μM forskolin (FSK), 1 mM 8-bromo-cAMP (8BA), 3 μM MG 132 (MG), 30 μM calpain inhibitor I (CPI), 30 μM lactacystin (LTC), 3 μM cycloheximide (CHX), 3 μM nitrendipine (NTR), and 1 μM Bay K 8644 (BK). Data (except those marked from Fig. 3) are the means from one or two experiments.

[a] From Fig. 3.

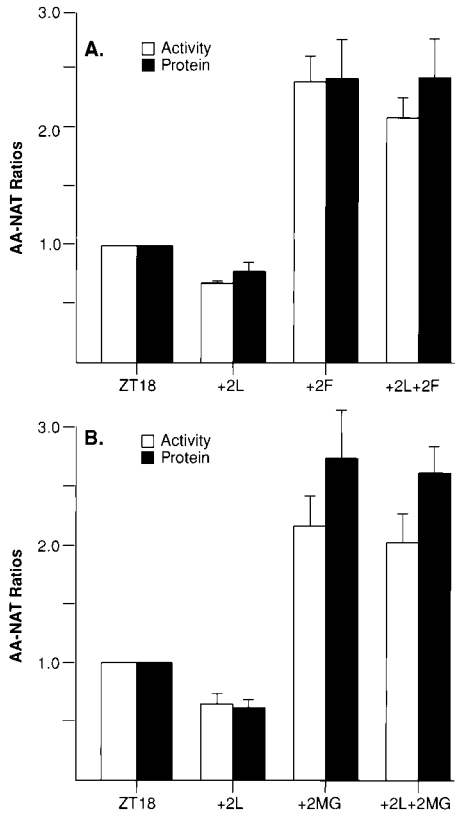

FIG. 3. Effects of light, forskolin, and MG 132 on AA-NAT protein levels and enzyme activity. **A:** Forskolin treatment strongly increased enzyme activity and protein levels and prevented or overcame the reductions seen with light. AA-NAT protein levels and enzyme activity were determined in cells harvested at midnight (ZT 18) in R, or after 2 h of exposure to L (+2L) or to 1 μM forskolin (+2F) or to both L and forskolin (+2L+2F) from ZT 16 to 18. Results are means ± SE from four experiments, each in duplicate, normalized to the midnight group (ZT18, taken as 1.0). **B:** MG 132 treatment strongly increased enzyme activity and protein levels and prevented or overcame the reduction seen with light. AA-NAT protein levels and enzyme activity were determined in cells harvested at midnight (ZT 18) in R, or after 2 h of exposure to L (+2L) or to 3 μM MG 132 (+2MG) or to both L and MG (+2L+2MG) from ZT 16 to 18. Results are means ± SE from four experiments, each in duplicate, normalized to the midnight group (ZT18, taken as 1.0).

Previous studies with protein synthesis inhibitors indicated that ongoing protein synthesis is required for the nocturnal increase in AA-NAT enzyme activity in both the rat and chick pineal, and for the response to cAMP (Klein et al., 1997). Recent studies in rat pineal directly measuring AA-NAT protein levels and the effects of various "protective" agents, however, have shifted emphasis from the promotion of synthesis to the prevention of degradation in the regulation of AA-NAT enzyme activity (Gastel et al., 1998). Those experiments indicated that AA-NAT protein is degraded by proteosomal proteolysis. We obtained similar results in the present studies using chick pineal cells. A 2-h treatment with the proteosomal proteolysis inhibitor, MG 132 (Palombella et al., 1994), increased both nocturnal AA-NAT protein levels and AA-NAT enzyme activity in parallel, and prevented or overcame the acute reductions seen with light (Fig. 3B). As with forskolin, similar (although proportionately larger) results were obtained with treatment of daytime cells (Table 1). Other proteosomal protease inhibitors, calpain inhibitor I and lactacystin (Fenteany et al., 1995), also increased AA-NAT protein levels and AA-NAT enzyme activity in parallel (Table 1).

DISCUSSION

The results of these experiments demonstrate that there is a daily rhythm in the levels of AA-NAT protein, as well as in enzyme activity, in cultured chick pineal cells. The persistence of the rhythm in RR indicates that it is truly circadian, even in vitro. In view of the similarity between the amplitudes of the rhythms in AA-NAT mRNA (Bernard et al., 1997*b*), AA-NAT protein levels, and AA-NAT enzyme activity, it appears that it is the circadian mRNA rhythm that drives the circadian rhythm in AA-NAT protein. In contrast, cAMP appears to act on enzyme activity primarily via its "direct" effect on AA-NAT protein levels in chick pineal cells, downstream from mRNA. Our findings also show that AA-NAT protein levels can be increased if proteosomal proteolysis is prevented. This evidence indicates that AA-NAT activity is regulated at at least two levels, at the mRNA and at the protein level, and is consistent with results in mammalian pineals. However, it is becoming increasingly clear that the relative importance of each mechanism varies from species to species (Klein et al., 1997, 1998) and between pineal and retina (Grève et al., 1999). For example, regulation at the protein level is the dominant regulatory mechanism in sheep: mRNA levels do not vary significantly with stimulation or time of day. However, in the rat, there are large mRNA responses to cAMP that play a major role in the timing and amplitude of the AA-NAT protein rhythm. In all cases, it appears that rapid light-induced changes in AA-NAT activity reflect changes in protein levels, and that these may be regulated through control of proteosomal proteolysis.

The presence of two immunoreactive bands in the chick pineal cells (Fig. 1A) is of interest, because it has not been seen in other systems. These bands varied in parallel under various conditions, suggesting that their regulation is essentially similar and that they are unlikely to represent unrelated proteins. Although further investigation will be necessary to understand fully the functional significance of these bands, it is reasonable to combine the two bands into a single measurement for the purposes of these experiments. This approach gave coherent and interpretable results, which support the hypothesis that there is a close correlation between AA-NAT protein and enzyme activity.

As stated in the introductory section, light acts on chick pineal AA-NAT activity in two ways: to acutely suppress its nocturnal levels and to entrain its rhythm. Previous studies have indicated that the acute suppres-

sive effect, in contrast to the entraining effect, is correlated with a decrease in cAMP (Zatz, 1996) and that agents affecting cAMP, including light, have a disproportionately small effect on mRNA levels relative to their effect on enzyme activity (Bernard et al., 1997*a*,*b*). Those results suggested that cAMP acts posttranscriptionally. If cAMP acted via phosphorylation or other posttranslational modification of AA-NAT protein, or by controlling the level of activating or inhibiting heterodimer, changes in AA-NAT protein levels would not have been apparent. However, we found that all agents that acutely change AA-NAT enzyme activity, including those that decrease cAMP levels (light pulses, norepinephrine) or intracellular calcium levels (nitrendipine), or raise cAMP levels (forskolin, 8-bromo-cAMP) or intracellular calcium levels (Bay K 8644), have a similar and parallel effect on AA-NAT protein levels and on AA-NAT enzyme activity. The drugs affecting calcium flux used here have been shown to affect cAMP levels (Zatz, 1992), but might also act via additional mechanisms, including more direct effects on AA-NAT proteolysis. The strong correlation between the effects of these agents on protein levels and on enzyme activity (but not on mRNA levels) indicates that cAMP plays an important role in regulating AA-NAT protein, and thereby regulates large and rapid changes in AA-NAT activity in the chick pineal.

The question arises whether the effects of cAMP on AA-NAT protein levels occur primarily at the translational or posttranslational level. It has long been known that elevating cAMP levels can slow the rate of loss of enzyme activity in the rat pineal, even in the absence of new protein synthesis (Deguchi and Axelrod, 1972; Romero et al., 1975), and it has been shown recently (Gastel et al., 1998) that this and other acute effects of changing cAMP levels result from changes in protein levels. Furthermore, evidence indicated that these changes in protein levels derived primarily from changes in rates of degradation via proteolysis (see below). Results reported here indicate similar changes in protein levels in chick pineal cells, and support a role for regulation of degradation through proteolysis. However, the slower and smaller acute decline of AA-NAT protein and enzyme activity in chick pineal cells, compared with that in rat pineal, makes it more difficult to rule out additional regulation at the translational level in the chick pineal.

In the rat pineal, inhibitors of proteosomal proteases increased AA-NAT protein levels and enzyme activity and prevented their reduction by propranolol (Gastel et al., 1998). Similar increases and protection against the effects of L (comparable to those obtained with forskolin) were observed in chick pineal cells (Fig. 3, Table 1). Thus, cAMP appears to regulate AA-NAT activity, as it does in the rat pineal, through inhibition of proteosomal proteolysis of AA-NAT protein. This action of cAMP may be more conserved among vertebrates than is the transcriptional regulation also provided by cAMP in the rat pineal. Certainly, it appears to be cAMP's most prominent action in chick pineal, where cAMP's transcriptional effect is relatively small. Such acute regulation of AA-NAT protein degradation can rapidly modify the enzyme activity provided by the chick pineal's more slowly changing circadian cycle of AA-NAT mRNA.

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